Characterisation of Lysozyme-Steroid Glucuronide Conjugates

A thesis presented in partial fulfilment of
the requirements for the degree of
Doctor of Philosophy in Chemistry
at Massey University.

Christopher Mark Smales

February, 1997

Abstract

The steroid glucuronides estrone glucuronide and pregnanediol glucuronide were synthesised using the O-glycosylation reaction of a glycosyl donor with the appropriate steroid under standard Koenigs-Knorr conditions. X-ray crystallographic studies showed that the synthetic estrone glucuronide molecule had the correct stereochemistry. Estrone glucuronide and pregnanediol glucuronide conjugates of hen egg white lysozyme were prepared by both the mixed anhydride and active ester coupling procedures. Both methods gave good yields of conjugate but the active ester procedure gave a more diverse range of products. Unreacted lysozyme, which was present in all cases, was removed by a combination of cation-exchange and hydrophobic-interaction chromatography to give purified conjugate material whose lytic activity was inhibited by over 90% in the presence of excess anti-steroid glucuronide antibody. Steroid glucuronide-lysozyme conjugates purified in this way could be used in a homogeneous enzyme immunoassay system to measure the levels of urinary estrone and pregnanediol glucuronide encountered in a normal menstrual cycle. Chromatography of the conjugation reaction mixtures on an S-Sepharose (fast flow) column in the presence of 7 M urea allowed the isolation of the different conjugate products. Conjugation of lysozyme with estrone glucuronide by the mixed anhydride method gave one major derivative exclusively acylated at lysine residue 33 while acylation with pregnanediol glucuronide gave two major derivatives exclusively acylated at lysine residues 33 and 97 respectively. On the other hand, conjugation of lysozyme with the two steroid glucuronides by the active ester method gave six derivatives which were acylated at combinations of one or more of three lysine residues, 33, 97, and 116. The correlation of the protein environments of the lysine amino groups in the crystal structure of lysozyme with the acylation positions in the conjugate families suggested that these positions were determined not only by the surface accessibility and nucleophilicity of the lysine residues but also by the steroid glucuronide and the acylating reagent. Computer derived three dimensional structures of the estrone glucuronide-lysozyme and pregnanediol glucuronide-lysozyme conjugates suggested that the enzyme conjugate may be inactivated by the antibody in the immune complex by either providing a physical barrier to approach by the large bacterial substrate or by disrupting the binding of the bacterial cell wall polymer into the active site cleft. The lytic activity of the estrone glucuronide-lysozyme E3 conjugate was not inhibited in the presence of excess antibody when the small chitohexaose substrate was used, implying that the substrate could access the active site even when the conjugate was bound by antibody. The detailed characterisation of the mixed anhydride estrone glucuronide-lysozyme conjugate coupled with the current knowledge of the antigenic determinants of hen egg white lysozyme has made it possible to design, in principle, a novel sandwich solid phase immunoassay format for the measurement of estrone glucuronide levels.

Acknowledgements

A large number of people have given me their time and advise during this thesis. As I can not mention everyone, thankyou to those of you not referred to below.

I am particularly indebted to my supervisor Associate Professor Len Blackwell for his support and guidance throughout the study presented (and for reading this thesis several times). Thanks must also go to Dr. Chris Moore (my second supervisor) who retired during the course of this study and Dr. Gill Norris for all her help and advice with crystallographic studies.

Others who have helped during this thesis that I wish to thank include;

Dr. Bryan Anderson for his help with Silicon Graphics modelling,

Associate Professor Dave Harding and Mr Dick Poll with chromatographic apparatus and advice,

Dr. Tony Burrell and Associate Professor Joyce Waters for solving the estrone glucuronide crystal structure,

Dr. Phil Jeffrey for solving the estrone glucuronide-lysozyme conjugate crystal structure,

Mr Dave Elgar for his advice on electrophoresis and related techniques, and

Dr. Roger Collin for his help with solid phase immunoassay colour test strips.

I must also thank all of the technical staff who have helped me during this study and the staff in stores who have always endeavoured to meet my needs. Of course such a study would be very dull indeed if it was not for the help, advice, humour and banter of my fellow postgraduate students within the Ovarian Monitor research group and the Departments of Chemistry and Biochemistry.

I am grateful to Massey University for a Massey University Postgraduate Scholarship.

A big thanks must also go to Mum and Dad who have supported me throughout my Ph.D. Finally, I would like to thank my wife Virginia to whom I am especially grateful to for all her support, help, and for putting up with the many highs and lows that come with scientific research.

Acknowledgement of Published Work

The following work has thus far been published from that presented in this thesis:

Smales, C. M., Cooke, D., and Blackwell, L. F. (1994) Use of ion-exchange and hydrophobic-interaction chromatography for the rapid purification of lysozyme-estrone glucuronide conjugates, *J. Chrom. B* **662,** 3-14.

Smales, C. M., Blackwell, L. F., Waters, J. W., and Burrell, A. K. (1997) Crystal structure of an estrone glucuronide hydrate, Submitted to *Acta Cryst.*

Table of Contents

Chapter Two

The Preparation and Purification of Estrone Glucuronide- and Pregnanediol Glucuronide-Lysozyme Conjugates

Conjugates Suitable for Use in a Homogeneous Enzyme Immunoassay System for the Measurement of Menstrual Cycle Levels of Urinary Estrone Glucuronide and Pregnanediol Glucuronide

---

Chapter Three

The Characterisation of Estrone Glucuronide- and Pregnanediol Glucuronide-Lysozyme Conjugates

---

Chapter Four

Structural Aspects of the Steroid Glucuronide-Lysozyme Conjugate-Anti-Hapten Antibody Immune Complexes

---

Chapter Five

The Kinetics and Mechanism of the Estrone Glucuronide-Lysozyme-Anti-Estrone Glucuronide Antibody Binding Reaction

---

Chapter Six

Studies Toward the Development of a Colour Test Immunoassay For the Detection of the Fertile Period

---

Chapter Seven

Crystallographic Studies

List of Figures

List of Tables

List of Schemes

Abbreviations

A_{280}	Absorbance at 280 nm
AB	Antibody
AE	Active ester
BBT	Basal body temperature
α-bromosugar	Methyl 1-bromo-1-deoxy-2,3,4-tri-O-acetyl-α-D-glucopyran-uronate
BSA	Bovine serum albumin
C_B	Concentration of bound conjugate
C_F	Concentration of free conjugate
C_T	Total conjugate concentration
CDR	Complementary determining region
Conj	Lysozyme-steroid glucuronide conjugate
DCC	Dicyclohexylcarbodiimide
DMF	Dimethylformamide
DMPTU	N,N-dimethyl-N'-phenylthiourea
E1G	Estrone glucuronide
E1G-AB	Anti-estrone glucuronide antibody
E1G[H]	Estrone glucuronide (acid form)
E1G-HEWL	Estrone glucuronide-hen egg white lysozyme conjugate
E1G-HRP	Estrone glucuronide-horseradish peroxidase conjugate
E2-17β-3G	Estradiol-17β-3-glucuronide
E3-3G	Estriol-3-glucuronide
E3-16G	Estriol-16α-glucuronide
E3-17G	Estriol-17β-glucuronide
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
EDTA	Ethylenediamine tetra-acetic acid
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assays
EMIT	Enzyme-multiplied immunoassay technique
EPPS	N-(2-hydroxyethyl)piperazine-N'-(3-propane sulfonic acid)
Fab	Antigen binding fragment
FPLC	Fast protein liquid chromatography
FSH	Follicle stimulating hormone
GlcNAc	N-acetylglucosamine
$(GlcNAc)_2$	Di-N-acetyl-chitobiose
$(GlcNAc)_3$	Tri-N-acetyl-chitotriose
$(GlcNAc)_4$	Tetra-N-acetyl-chitotetraose
$(GlcNAc)_5$	Penta-N-acetyl-chitopentaose

$(GlcNAc)_6$	Hexa-N-acetyl-chitohexaose
GnRH	Gonadotropin releasing hormone
HCG	Human chorionic gonadotrophin
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethane sulfonic acid)
HEWL	Hen egg white lysozyme
HEWL-AB	Anti-hen egg white lysozyme antibody
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
HRP-E1G	Horseradish peroxidase-estrone glucuronide conjugate
I.D.	Internal diameter
IgG	Immunoglobulin G class of antibody
LH	Luteinizing hormone
MA	Mixed anhydride
MCAB	Estrone glucuronide monoclonal antibody
m.p.	Melting point
MurNAc	N-acetylmuramic acid
NAD^+	Nicotinamide adenine dinucleotide phosphate (oxidised form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NAG	N-acetylglucosamine
NAM	N-acetylmuramic acid
NFP	Natural Family Planning
NHS	N-hydroxysuccinimide
NMR	Nuclear magnetic resonance
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PdG	Pregnanediol glucuronide
PdG[H]	Pregnanediol glucuronide (acid form)
PEG	Polyethylene glycol
PIPES	Piperazine-N,N'-bis-(2-ethane sulphonic acid)
PITC	Phenylisothiocyanate
RIA	Radioimmunoassay
r.m.s.	Root mean square
µs	Microsphere
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
ΔT	Change in transmission
TFA	Trifluoroacetic acid
TMB	Tetramethylbenzidine
TNBS	2,4,6-trinitrobenzene 1-sulfonic acid

tris	Tris(hydroxymethyl)aminomethane
WHO	World Health Organisation

Amino Acid Abbreviations

A	Ala	Alanine
C†		S-carboxymethylated cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
I	Ile	Isoleucine
K	Lys	Lysine
K*		Steroid glucuronide acylated lysine residue
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine